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ABSTRACT

Background : Bardet-Biedl syndrome (BBS) is a genetically heterogeneous, multisystemic disorder characterized by progressive retinal dystrophy, obesity, hypogenitalism, learning difficulties, renal abnormalities and postaxial polydactyly, with only the last two antenatally observable. BBS is inherited as an autosomal recessive disorder and 14 genes have been identified to date (*BBS1-BBS14*). In addition, a complex digenic inheritance has been established in some families. Mutations of the *BBS10* gene on chromosome 12q21.2 account for 20% of BBS cases.

Methods: Given the fact that mutations in BBS genes have already been found in Meckel-like fetuses, and in light of the major contribution of *BBS10* to BBS, we sequenced the *BBS10* gene in 20 fetal cases and a child diagnosed antenatally presenting characteristic renal anomalies and polydactyly, but without biliary dysgenesis.

Results: We identified recessive mutations at the *BBS10* locus in 5 cases, 4 fetuses and a child. Interestingly, one of them had *situs ambiguus*, a rare feature in BBS. In the child, *BBS* genes screening identified a heterozygous *BBS6* nonsense mutation in addition to the homozygous *BBS10* mutation, in accordance with the suggested multigenic inheritance of the disease.

Conclusions: These results confirm that BBS is underdiagnosed antenatally, and should systematically be suspected in fetuses with severe cystic kidneys leading to oligoamnios and fetal or perinatal death. Moreover, this study confirms the high frequency of *BBS10* mutations and particularly of the p.Cys91Leufs*5 allele, including in severe lethal cases.

INTRODUCTION

Congenital anomalies of the kidney and urinary tract (CAKUT) in association with non-renal anomalies are found in more than 200 syndromes. CAKUT represent a broad range of disorders that result from abnormal embryogenic renal development due to parenchymal malformations, abnormalities in migration of organ primordia, or abnormalities in the developing collecting system. Malformations of the renal parenchyme lead to failure of normal nephron development in terms of renal dysplasia, renal agenesis, renal tubular dysgenesis, and polycystic renal diseases. Cystic kidneys can be detected on prenatal ultrasonography, particularly during the second trimester, and can cause fetal death *in utero* by oligohydramnios or associated anomalies. This feature, visible on ultrasound, can appear isolated, as in polycystic kidney disease, or associated with multiple congenital anomalies. Accurate diagnosis in antenatal cases is important to evaluate prognosis, and to propose adequate management of the pregnancy and genetic counselling.[1]

Bardet-Biedl syndrome (BBS, OMIM 209900), initially described by Bardet in 1920 and Biedl in 1922, is an autosomal recessive multisystemic disorder characterized by progressive retinal dystrophy, postaxial polydactyly, obesity, hypogenitalism, learning difficulties and renal abnormalities. In addition to the major diagnostic features, multiple other manifestations have also been documented.[2, 3] These include diabetes mellitus, neurological signs, behavioural traits, facial dysmorphism, dental anomalies, heart disease, and hepatic fibrosis.[3] Prevalence rates in North America and Europe range from 1:125,000 to 1:175,000 live births.[2] Because of the late onset of symptoms, the diagnosis of BBS is usually made during childhood and certainly underdiagnosed antenatally. At birth, suggestive features are polydactyly, renal abnormalities, genital or heart malformations. However, it has been shown that a severe clinical presentation of BBS including lethal cystic kidneys disease can mimic Meckel Syndrome (MKS, OMIM 249000). [4]

Fourteen *BBS* genes have been identified to date (*BBS1-BBS14*). Two of these genes, *MKS1 (BBS13)* and *CEP290 (BBS14)* are usually involved in Meckel Syndrome and Joubert Syndrome, and may have a potential epistatic effect with known BBS-associated loci.[5] *BBS* genes encode proteins involved in the development and function of primary cilia.[6] BBS therefore became a model to help understand of ciliary signalling mechanisms.[7] This discovery of other disorders caused by similar ciliary structural and signalling defects supported the concept of "ciliopathies"[8], inherited diseases resulting from ciliary dysfunction.[9] Ciliopathies comprise phenotypically overlapping disorders [10] with renal malformations (renal dysplasia and cystic kidney disease) being one of the more common unifying features[11, 12] and a variable degree of polydactyly, obesity, hypogonadism, central nervous anomalies, retinitis pigmentosa and laterality defects.

Mutations in one of the 14 known *BBS* genes are found in approximately 80% of BBS cases.[7] In addition, a complex digenic inheritance has been established in some families, where three mutations at two *BBS* loci are necessary for the expression of the disease.[8, 13-15]. The *BBS10* gene, also called *FLJ23560*, identified in 2006 by Stoetzel *et al*., accounts for 21% of BBS cases.[16] *BBS10* encodes a vertebrate-specific chaperonin-like protein which interacts with bbs1, 4 and 6 in zebrafish[16] and is localised at the basal body of the primary cilium.[17] Recent studies have demonstrated the role for the three chaperonin-like BBS proteins (BBS10 with BBS6 and BBS12) in forming a complex of CCT/TRiC family chaperonins which then mediates BBSome assembly, a structure which transports vesicles within the cilium.[18] Given the phenotypic overlap of ciliopathies, the fact that mutations in *BBS* genes have already been found in Meckel-like fetuses, and the major contribution of *BBS10* to BBS, we screened for mutations in *BBS10* in 20 fetuses and a child referred for cystic kidneys, polydactyly and/or a central nervous system anomaly but without the encephalocele or biliary dysgenesis characteristic of Meckel syndrome.

MATERIAL AND METHODS

We analysed the *BBS10* gene in 20 cases with cystic kidneys diagnosed antenatally and polydactyly, but without occipital encephalocele or biliary dysgenesis. Parental consents for the genetic study were obtained in all cases. In all but one, pregnancy was terminated in accordance with French legislation either because of the severe renal dysfunction (oligohydramnios) or because of its association with another anomaly. Chromosome analysis and clinicopathological examination were performed in all cases except case one, where parents declined pregnancy termination after genetic counselling. Clinical and histological features of mutated cases are summarized in Table 1.

Genomic DNA was extracted from frozen fetal tissues or from a blood sample in case 1, and from parents. Primers used were previously described.[16] All amplifications were performed under the same conditions, using a touchdown protocol consisting of denaturation for 30s at 96°C, annealing for 30s at a temperature ranging from 64°C to 50°C (decreasing 1° during 14 cycles, then 20 cycles at 50°) and extension at 72°C for 30s. PCR products were treated with ExoSAP-IT (USB, Affymetrix). Both strands were sequenced with the appropriate primer and the BigDye terminator cycle sequencing kit (Applied Biosystems, Foster City, CA) and analyzed on ABI3130 automated sequencers (Applied Biosystems, Foster City, CA). Segregation of the identified mutations was investigated in parents when available. Mutation numbering is based on cDNA sequence with a 'c.' symbol before the number, where +1 corresponds to the A of ATG translation codon (codon 1) of the cDNA reference sequences (NM_024685). Mutation names were checked by the Mutalyzer program.[19]

RESULTS

We found *BBS10* mutations in five cases, four fetuses and a child. Clinical and mutation data are presented in Table 1.

Table 1: Clinical data and *BBS10* mutations

Case	Age	Kidney	PD	Other	Nucleotide alterations	Exon	Predicted effect on protein
1	20y	PRF	UL,	Obesity	c.271dupT	2	p.Cys91LeufsX5
CRE			LF	PRD	c.271dupT	2	p.Cys91LeufsX5
					BBS6 mutation: p.Arg139X		
2	23w	MKS-like	RH,		c.271dupT	2	p.Cys91LeufsX5
LAN			RF		c.271dupT	2	p.Cys91LeufsX5
3	26w	MKS_like	4 L		c.271dupT	2	p.Cys91LeufsX5
ILL					c.1044-1045delTT	2	p.Pro350IlefsX11
4	21w	MKS_like	LL	*Situs ambiguus*, polysplenia, pancreas hypoplasia (absence of the tail), pulmonary artery stenosis	c.273C>G	2	p.Cys91Trp
EIW					c.1044-1045delTT	2	p.Pro350IlefsX11
5	22w	MKS_like	LL		c.185A>G	1	p.His62Arg
DEC					c.271dupT	2	p.Cys91LeufsX5

Foetal cases: age is given in gestational weeks. PRF: progressive renal failure, MKS-like: cystic kidneys similar to that observed in Meckel syndrome, PD: polydactyly, 4L: 4 limbs, UL: upper limbs, LL: lower limbs, RH: right hand, RF: right foot, LF: left foot, PRD: progressive retinal dystrophy.

Case 1 is now a twenty year-old young woman, seen antenatally because of the association of enlarged hyperechogenic kidneys and postaxial polydactyly, and described previously (case 7 of Karmous-Benailly).[4] After birth, the size of the kidneys decreased to normal while progressive renal failure appeared. Obesity started at 3 years and an electroretinogram examination established the diagnosis of BBS. At 12 years of age, vision was normal. Direct sequencing of the *BBS10* gene found a homozygous T insertion in exon 2, at residue 91, leading to premature protein termination (c.271dupT, p.Cys91LeufsX5). Moreover, previous sequencing of *BBS1 to BBS8* genes had revealed a heterozygous mutation in exon 3 of the *BBS6* gene: p.Arg139X.[4]

Case 2 was a 23-weeks old female fetus. The pregnancy was terminated because of severe cystic kidneys and anamnios. Autopsy revealed enlarged kidneys with nonetheless normal nephronogenesis, containing multiple thin-walled cysts along nephron segments and collecting ducts, and demonstrating a gradient in the size of cysts from periphery to center,. The parenchyme was otherwise normal, without metaplasic cartilage. The kidneys were therefore histologically similar to those observed in Meckel syndrome, and termed “Meckel-like” kidneys (Fig 1A). The

fetus had also right hand and foot hexadactyly. No other abnormality was found, in particular no liver anomaly (Fig 1B). Molecular studies found a homozygous frameshift mutation in exon 2 c.271dupT, p.Cys91LeufsX5.

Case 3 was a 26-week old female fetus presenting enlarged hyperechogenic kidneys and hexadactyly of all four limbs. Meckel or Bardet-Biedl syndromes were evoked antenatally. After genetic counselling, parents asked for termination of pregnancy. Autopsy showed facial dysmorphism with a round face, hypertelorism, small flattened nose, micro-retrognathism and small ears, characteristic of Potter's dysmorphism. Examination confirmed post-axial hexadactyly and cystic kidneys. Histological examination of the kidneys showed "Meckel-like" cyst size and number augmentation from the cortex to the medulla (Fig 1C). Lesions were moderate with persistence of cortico-medulary differentiation and sub-capsular nephrogenesis. The liver was histologically normal (fig 1D). Direct sequencing of *BBS10* found compound heterozygous mutations. The fetus carried a T insertion at residue 91 leading to premature termination c.271dupT, p.Cys91Leufs*5 (fig 1I) inherited from his mother and a 2 base pair deletion paternally inherited: c.1044_1045delTT, p.Pro350Ilefs*11 (fig 1J).

Case 4 was a 21-weeks old male fetus. The pregnancy was terminated because of the association of cystic kidneys and *situs ambiguus*. Examination of this fetus found a facial dysmorphism with retrognatism and hypoplastic nasal bone characteristic of Potter sequence. There were postaxial foot polydactyly, pulmonary artery stenosis, and *situs ambiguus* where the stomach and spleen were localized on the right side with polysplenia. The pancreas was hypoplasic with absence of the tail**.** Histological examination of the kidneys showed Meckel-like cyst size and number augmentation from the cortex to the medulla (Fig 1E). Pancreatic ducts were slightly dilated. There were no brain or liver (Fig 1F) anomalies. Direct sequencing of *BBS10* found two heterozygous mutations: a missense mutation c.273C>G, p.Cys91Trp (fig 1K) inherited from his mother and a TT deletion in exon 2 c.1044-1045delTT, p.Pro350Ilefs*11.

Case 5 was a 22 week female fetus presenting Meckel-like cystic kidneys (fig 1G), lower limb postaxial polydactyly and a Potter sequence with dysmorphism and contracted limbs. The liver was normal (Fig 1H). A previous pregnancy was terminated because of cystic kidneys and hexadactyly 11 years ago. We identified two heterozygous mutations: a maternal missense mutation c.185A>G p.His62Arg located in exon 1 (Fig 1L) and the recurrent c.271dupT, Cys91Leufs*5 inherited from the father.

Among the 15 other cases in this series, eight had mutations in another *BBS* gene, namely *BBS2* (3 cases), *BBS4* (2 cases), *BBS6* (2 cases) and *BBS7* (1 case), and six of them have been reported previously.[4] In addition, two fetuses within this series had agenesis of the cerebellar vermis, and in these, mutations were found in the *CEP290* gene (*NPHP6/MKS4*), where biliary dysgenesis is known to potentially be absent. These two cases have also been described previously (cases 27 and 29).[20] In the five remaining cases, all with similar Meckel-like kidney histology, polydactyly, but without biliary dysgenesis or brain malformations, no *BBS* gene mutations have been identified thus far.

DISCUSSION

Genetic screening in the cohort

The most characteristic diagnostic criteria for BBS, such as obesity and retinal anomalies, appear postnatally. Only the renal abnormalities, congenital heart defect, genital anomalies and polydactyly can be detected antenatally. In particular, enlarged and undifferentiated hyperechogenic kidneys on prenatal ultrasonography may suggest the diagnosis of BBS.[21] A previous study showed that renal histological anomalies in BBS fetuses can be very comparable to those observed in Meckel Syndrome.[4] However, liver histological anomalies included hepatic fibrosis but not biliary dysgenesis. Based on our previous analysis of *BBS* genes in antenatal cases, and the major contribution of *BBS10* to BBS, we focused on a series of cases with cystic kidneys and polydactyly

but no biliary dysgenesis to evaluate the contribution of *BBS10* to this association. *BBS10* mutations were found in 5/20 cases, demonstrating that *BBS10* mutations are also observed in the lethal form of the disorder and that the c.271dupT, p.Cys91Leufs*5 mutation is a major contributor to the fetal form as well. Overall, 13/20 fetuses carried *BBS* gene mutations, further highlighting the recognized clinical variability that characterizes Bardet-Biedl syndrome (BBS). This frequency confirms that BBS can present as a perinatal, even lethal form, and suggests that the combination of renal malformation and polydactyly alone can be highly predictive of BBS.

BBS10 mutated alleles and oligogenism

The same c.271dupT, p.Cys91Leufs*5 was found at the homozygous state in two non consanguineous and unrelated cases (cases 1 and 2), and represented 6/10 of the mutated alleles in our series. This is in accordance with the previous reports, where missense and truncating *BBS10* mutations have been found in 21% of BBS patients, the p.Cys91Leufs*5 change accounting for 46% of the mutant alleles in the report of Stoetzel.[16] This recurrent mutation is therefore the cause of the major contribution of *BBS10* to the disease. Whether this mutation represents an ancient allele or a mutational hotspot has been debated, but the second hypothesis is favoured because of its identification in populations of various origins. Interestingly, unrelated cases 3 and 4 were compound heterozygous for *BBS10* mutations, both carrying the same c.1044_1045delTT, p.Pro350Ilefs*11 mutation. This mutation has also been reported twice by Stoetzel *et al.* among the 118 *BBS10*-mutated alleles in their series.[16]

In case 1, in addition to the homozygous *BBS10* mutation, a heterozygous nonsense *BBS6* mutation was observed, in accordance with the suggested oligogenic inheritance of BBS. Indeed, in some families, three mutations at two loci are necessary to cause this disease.[13, 14] Such observations provide a clue to the clinical variability of BBS[8, 13], since in some families, two mutations at one locus have been sufficient to cause BBS, but a third mutation at another locus has been found in a more severely affected sib.[8, 13] Moreover, a single gene can have either a causal or a modifying role in the disease.[14] The ability of each *BBS* locus to modify the phenotypic expression

of mutations in another *BBS* gene first suggested they are part of a same multiproteic complex, which has been demonstrated since by the identification of at least seven BBS proteins within a complex known as the BBSome, involved in ciliary protein trafficking[22] Moreover, BBS6, BBS10, and BBS12 form a complex and are involved in BBSome assembly.[18] The complex inheritance is relevant in light of genetic counselling and the explanation of variable expressivity. *BBS6* is more often associated with oligogenic inheritance than the other *BBS* genes, as heterozygous mutations are the most frequent.[8]

BBS10 phenotype

The clinical presentation of these five cases is consistent with previously described antenatal cases of BBS. In particular, no brain anomalies were found. One of our cases, Case 4, presented other features, namely *situs ambiguous* with polysplenia, pancreatic hypoplasia (absence of the tail) and pulmonary artery stenosis.

Other studies of BBS have described less frequent clinical signs, including laterality defects such as *situs inversus* in a few cases [23, 24]. Ansley described 3 affected siblings with a homozygous 3 bp deletion in the *BBS8* gene.[6] One of the siblings had complete *situs inversus*, pointing to variable expressivity of this feature. Moreover, Yen *et al*. demonstrated the implication of *BBS* genes in lateralization using knockdown zebrafish for the homologues of six human BBS genes, *BBS2, 4, 5, 6, 7* and *8.* The result is a disruption in Kuppfer's vesicle, a ciliated organ playing a role in lateralization during embryogenesis.[25] Deffert *et al*. proposed that *situs inversus* could be considered to be a minor criterion for BBS. However, this criterion is a rare but non-specific feature of many ciliopathies.

Pleiotropic effect of ciliopathies : common mechanisms

The clinical phenotype of BBS is extremely variable and overlaps with other ciliopathies. There is also a genetic overlap, as the same gene mutations can be involved in different ciliopathies.[5, 20, 26-28] Common features can be explained by common pathways, protein interactions,

or multi-subunit complexes.[12, 28] Primary cilia are present in numerous cell types and are involved in many cellular functions as cell polarity, chemosensation and mechanosensation, intercellular junctions' formation, cell-cell contacts and interactions with the extracellular environment.[29] Pleiotropic features of ciliopathies are explained by the interference with signalling pathways necessary for embryonic development of organ primordia. Components of the ciliary signaling machinery that have been identified include ligands and effectors of the Hedgehog[30], Wnt[31], and Platelet-Derived Growth Factor Receptor[32] signaling pathways. Following signal reception, ciliary transduction controls the balance between cellular differentiation, cell division and apoptosis through the regulation of such key developmental pathways.[33]

Genotype - phenotype correlations in ciliopathies

While there is a clinical and genetic overlap between ciliopathies, differences in severity can be explained by the nature of the mutation itself, for some genes. For example, Delous et al. showed that while *RPGRIP1L* missense mutations lead to JBS, truncating mutations lead to MKS.[28, 34] A strong genotype-phenotype correlation was also shown for the *CC2D2A* gene (*JBS9, MKS6).*[35] These data suggest that the two phenotypes represent a continuum in the spectrum of a single disorder. Leitch *et al*. showed that hypomorphic mutations in the *MKS1* gene can be associated with BBS, while, to date, only *MKS1* truncating mutations have been found in MKS fetuses.[5] Other MKS genes may act as modifiers or be directly implied in digenic transmission.[5] Therefore, in other instances, when no evident genotype-phenotype correlations are found, clinical variability might be explained by the total mutational load in ciliopathy genes, as suggested by the oligogenic inheritance in Joubert/MKS, nephronophthisis[36] and retinal degeneration of ciliopathies.[37]

Conclusion

This study suggests a high frequency of *BBS10* mutations in the severe antenatal and lethal form of BBS, a frequency also observed in postnatal cases. Overall, previous analyses of *BBS1-8* genes and the new data brought by this study strongly support the conclusion that the association of

renal anomalies and polydactyly, without biliary dysgenesis or brain anomalies, can be highly predictive of Bardet Biedl before birth (13/18, 70%). One-fifth of BBS cases still remain unaccounted for by mutations in the 14 loci described to date. Our data support the existence of other, yet unidentified, *BBS* genes to explain our failure to find mutations in certain suggestive antenatal cases. The identification of *BBS* genes and the delineation of a fetal phenotype of BBS has improved the molecular diagnosis of lethal cystic kidney disease before birth and allows vastly improved genetic counselling for concerned families.

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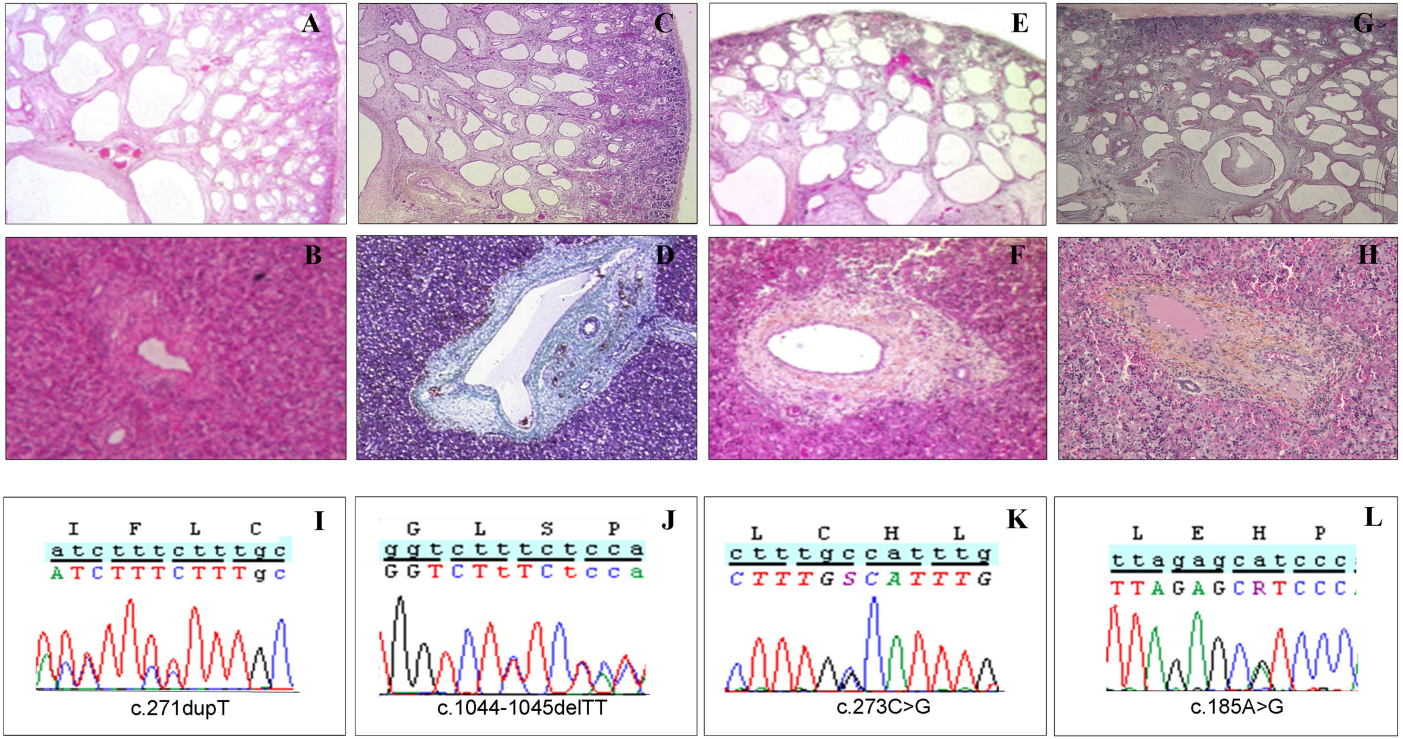
LEGENDS OF FIGURES

Figure 1: A-H: Histological sections of kidney (A, C, E, G) **and liver** (B, D, F, H) of four fetuses carrying *BBS10* mutations. The four cases show kidney anomalies usually found in Meckel syndrome with normal nephrogenesis, thin-walled cysts along various nephron segments and collecting ducts, and a gradient in the size of cysts from the cortex to the medulla. The parenchyme was otherwise normal without metaplasic cartilage. There is no biliary dysgenesis. **I-L: Sequence chromatograms:** Sequence traces from cases 1 to 5: c.271dupT; p.Cys91LeufsX5 (I), c.1044-1045delTT; p.Pro350Ilefs*11 (J), c.273C>G; p.Cys91Trp (K) and c.185A>G; p.His62Arg (L).


Case 2
Case 3
Case 4
Case 5
A
C
E
G
B
D
F
H
I
I F L C
atctttctttgc
ATCTTTCTTTgc
c.271dupT
J
G L S P
ggtctttctcca
GGTCTtTCtcca
c.1044-1045delTT
K
L C H L
ctttgccatttg
CTTTGSCATTTG
c.273C>G
L
L E H P
ttagagcatccc
TTAGAGCRTCCC
c.185A>G